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APPLICANT: MANSOUR SAMADPOUR

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Dr. Frank W. Lu
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I respectfully submit the following itemized response to the Detailed Action regarding the above reference application. Responses are numbered, and each number corresponds to your itemized statements in the Detailed Action Document. Please note that the Du Pont Legal Division no longer represents me in the following application. I am preparing this response on December 20

a. Article # 3 Drawings
A set of figures were prepared and were to have been submitted with the original application. I have enclosed copies of the figures with this response. I will ask for the originals from Du Pont Legal Division to be sent to you directly.

b. Article #4: Abstract
I have enclosed an abstract sheet with this response.

c. Rejection of claims 7, 8, 10, and 17 under U.S.C. 112, first paragraph.
I respectfully request that the claims be reinstated. The method, Micro Restriction Fingerprinting (MRF), can be used to cut the genomic DNA into fragments ranging from 0.1 to 18 kb. The exact size range depends on the specific genomic DNA and the restriction enzyme. While the lower size range is at 0.1 kb range the upper size could vary from a few killobases to 18 kb. In any MRF subtyping all or a portion of the resulting fragments can be analyzed to identify genetic subtypes. Claims 7, 8, and 10 refer to the use of electrophoresis methods for resolving the MRF generated fragments. For a person skilled in the art, one does not need to specify details of various gel electrophoresis methods. Anyone skilled in the art can easily use PFGE , horizontal gel electrophoresis, field inversion or capillary gel electrophoresis for resolving DNA fragments. The choice of the method depends on

the fragment size range that they will decide to resolve.

PFGE can be used to resolve small DNA fragment sizes. The most commonly used PFGE equipment sold by Bio Rad, Cheff Mapper, can be set to separate any size range. The fact that the PFGE can be used to resolve large fragment sizes does not mean that it can not be used to resolve small fragment ranges. Specifically, regarding the capillary electrophoresis, it can be used when the resulting RFLP patterns are in the lower ranges.
To a person skilled in the art it is obvious to use capillary gel electrophoresis for resolving smaller, PFGE and field inversion for larger size ranges, and one dimensional gel electrophoresis for small to medium size range. Claim 10 is for the use of various dyes for staining the DNA fragments for visualizing /detecting the bands. Again for a person skilled in the art, the use of DNA staining protocols is a rudimentary laboratory protocol which would not require directions or description. Conducting electrophoresis using various methods is so elementary to this field that giving instructions equates to providing instructions as to the use of hammers for carpenters.

d. Article 7-8: Rejection of claims 16-27 under 35 U.S.C. 112, second paragraph, for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

I respectfully ask that the claims be reinstated based on the following. While we agree that claims 17-27 are dependent on claim 16, claims 17-27 encompass the various ways that the MRF-RFLP can be generated, resolved, and visualized/detected. Claims 17-20 lists the various electrophoresis methods that can be used to resolve the RFLP patterns, the media for electrophoresis, and dyes for visualizing/detecting the RFLP patterns. Claim 21 and 22 refer to the restriction enzyme or combination of restriction enzymes that can be used to generated the MRF range (0.1-18 kb) RFLP patterns. Claim 23 refers to the quantity of the DNA that is to be cut to generated the MRF-RFLP pattern that can be easily visualized/detected. Claim 24 refers to the length of the electrophoresis time and the length of the gel, both of which can be manipulated to eliminate the smaller size range. Claim 25 refers to the electrophoresis conditions that would result in resolving the RFLP pattern over a distance to 4-12 Cm. Claims 26-27 refer specifically to microbial species that have been typed using the MRF method. Claims 17-27 aim to point out the variations of the MRF method, and as such the claims are valid and are an integral part of the patent. Claim number 16 will be discussed in the following section.

e. Articles 9 and 10: Rejection of claim 16 as vague and indefinite over the use of phrase "the lowest index of diversity", or "the highest index of diversity".

I respectfully request that the claim be reinstated for the following reasons. I preface by saying that your interpretation of the index of diversity is correct. The index of diversity can be calculated in a few different manners. The easiest and the most intuitive is the one that you used. In my description of the invention I stated that in determining the most suitable restriction enzyme for a new organism to be subtyped by MRF, we would put together two collections of isolates from the species in question. Lets say that we will assemble 20 isolates of species X from a cluster of cases in a waterborne outbreak, this will be our collection of epidemiologically linked isolates. We will also assemble a collection of isolates with no apparent epidemiological linkage (isolated at different times, from different sources, at different places). This will be our epidemiologically unlinked collection of isolates. Both collections will be subtypes with a battery of 20 restriction enzymes. Ideally we are looking for an enzyme(s) that would give the highest index of diversity for the epidemiologically unrelated isolates, and the lowest index of diversity for the epidemiologically linked collection of isolates. That would be an enzyme that would divide the 20 unlinked isolates into 20 groups, giving the ID= 20/20=1, and would give an identical RFLP pattern for the 20 isolates that are from the same outbreak, giving an ID of 1/20=0.05. The index of diversity ranges from >0 to 1.0. For a person well versed in the art the stated criteria is not vague nor is it indefinite. Any molecular epidemiologist would understand the criteria the way it is stated. The goal is to identify en enzyme that has the highest differentiative power for the epidemiologically unrelated collection of isolates, and would see the collection from an outbreak (epidemiologically linked) as identical or with the least diversity. Again anyone with ordinary skill in the art would have a complete understanding of the terms and the stated criteria.

f. Articles 11 and 12: Claim rejections based on 35 U.S.C. § 102; Claims 1-4, 6, 11, 12, 16, 19, 21, 25, and 26 are rejected as being anticipated by Preston et al., (J. Clin. Microbiology, 32, 1427-1430, 1994).

I respectfully request that the claims be reinstated based on the fact that none of the claims could have been anticipated from Preston et al. (1994).

The work by Preston et al. (1994) is the best example of prior art that could not have anticipated the invention. While it is obvious to anyone skilled in the art that most of the restriction enzymes with four base recognition sites cut the DNA in large number of small fragments, this did not result in developing a useful method for interstrain differentiation. Preston et al used a *Hae* III to generate RFLP patterns for *Shigella sonnei*. The size range of the patterns were from .2-4.3 kb. Preston could not resolve the fragments using agarose gel electrophoresis, or PFGE. He could not

stain the fragments using ethidium bromide, propidium iodide, and fluorescent dyes. The only way that he could visualize the banding pattern was to resort to silver staining which is a complicated staining process (takes 4-6 hours using a variety of toxic chemicals, vs. 10-20 minutes of staining using a dye). The quality of the banding pattern generated by Preston was such that his method was not used by others mainly due to difficulties in analysis because of poor quality of the RFLP patterns. With the MRF criteria stated in our invention neither *Hae*III nor *Rsa*I would qualify as the primary enzyme of choice for *Shigella sonnei*, We have identified *Sau*3A as the enzyme of choice for *S. sonnei* (page 19 of the application #10, and figure 3B. The difference between our methodologies are as follows:
If we were to subtype a collection of S. sonnei isolates the enzyme reaction would have been set using 10X more DNA than that used by the Preston et al, the enzyme used would have been Sau3A instead of HaeIII, the restricted DNA would have been resolved by agarose gel electrophoresis instead of polyacrylamide, and the gel would have been stained using a fluorescent dye instead of the silver stain, and the size range would have been .1-13 kb instead of .2-4.3. The result would have been easy to analyze because of the quality of the banding pattern (bright well separated bands). We urge you to look at the quality of the RFLP patterns generated by the Preston et al.'s method. This simple factor alone explains why the method was never adapted for use in the field. The MRF method generated RFLP patterns that have high differentiative power and can rival PFGE patterns in their quality. Like PFGE it is a universal typing method and we have shown its use for a large number of gram positive and gram negative bacteria. The method drastically different from the one by Preston et al. presto's work can not be viewed as foundational for development of MRF since it has nothing in common with the MRF method, furthermore despite a widespread need for a simple chromosomal based subtyping method and a large number of people in the field skilled in the art no one was able to use Preston's method or modify it into a useful method.

g. Articles 13 and 14: Claim rejections under - 35 U.S.C. § 103: Claims 5, 14, 15, 18, 24, and 27 are rejected as being unpatentable over Preston et al. (1994), and claims 1-4, 6, 11, 12, 16, 19, 21, 25, and 26 are not patentable over Samadpour et al (1993).

I respectfully ask that the claims be reinstated on the basis of these facts. I discussed the limitations of the Preston's method in the previous section Preston's method has completely different than the MRF method, and it was not reported for anything but Shigella, as such claims 4-5 should be reinstated.

Claim 14, 15, 16, and 23 should be reinstated since the cited reference (Nucleic acid hybridization: A practical approach, Hames and Higgins, 1985, pages 1456 and 146) is inappropriate. The reference protocol is for isolation and purification of DNA fragments (for making DNA probes or for cloning) using a preparative agarose or polyacrylamide gels. As you are well aware the width of the wells in preparative gels are 10-20 times that of analytical gels, that is why we can load 10-50 micrograms of restricted DNA on such gels without overloading the gels. This has nothing to do with the use of the 5-30 micrograms of DNA in MRF in wells which are 10-20 times narrower than the preparative gels. No one skilled in the art can make a jump from the referenced protocol into developing the MRF method. The use of 5-30 micrograms of DNA in subtyping reactions is counterintuitive and against all of the existing protocols. This was an unexpected result and discovery. Samadpour et al's (1993) does not void claims 13 and 14. In that work Samadpour used one microgram of DNA per reaction, as opposed to 5-30 micrograms, using one microgram for MRF will result in a RFLP pattern that can not be visualized, since the method generates a large number of small fragments, there simply is not enough of each of the fragments to allow for visualization using any of the staining protocols for agarose gels. The enzymes used by Samadpour were six base cutters in individual reactions. These enzymes can not be used in MRF since they generated fragments in 1-50 kb size range, which makes it impossible to analyze the resulting RFLP patterns (A very compact cluster of bands that often shows as a smear). That is why Samadpour had to resort to the use of southern blotting with subsequent probing using radioactive labeled lambda DNA probe to obtain an RFLP pattern that he was able to analyze. The MRF method circumvents the need for southern blotting and probing by generating a differentiative RFLP pattern with a simple restriction enzyme reaction followed by agarose gel electrophoresis.

I respectfully disagree with the assertion that the MRF method is a simple substitution of organism (E. coli instead of Shigella), and polyacrylamide with agarose. We have shown that the MRF method is universal to bacterial species, the method is based on the use of class of enzymes or combination of enzymes that can generated fragments below 18 kb, this allows for effective separation of the fragments using agarose gel electrophoresis or other forms of electrophoresis, we have determined that in order to be able to visualize the fragments using rapid and simple staining protocols, we need to use 5-30 times more DNA than is customarily used. We have invented and optimized a universal subtyping methodology that is the only viable alternative to PFGE subtyping, by being faster, cheaper, easier to perform and has more differentiative power. The discovery was quite unexpected, up to now when the work is presented in scientific circles the results are quite surprising for learned academics with mastery of the art. The unexpected part of the invention was that using 5-30 times the amount of DNA that

is restricted for subtyping reactions, and a number of enzymes that were not routinely used in chromosomal DNA fingerprinting, and simple gel electrophoresis units , agarose, and normal fluorescent stains we were able to generated RFLP patterns for any gram negative or gram positive bacteria in a course of 24 hours, that was as good or better than what was obtained using the PFGE method which requires electrophoresis units that cost $10,000-$20,000, and at the time of the invention would take five days to perform.

h. Article 15: rejection of claims 7, 9, 10, 17, and 20 as being unpatentable over Preston et al. (1994)as applied to claims 1-4, 6, 11, 12, 16, 19, 21, 25 and 26 above, and further in view of Arakawa et al., (1994).

Dr. Lu, I respectfully request that the claims be reinstated in view of the fact that the limitation of Preston's work was discussed in details above, and Arakawa's work dose not apply here. There is a big difference between the ability to separate and detect PCR amplicons and that of genomic DNA based RFLP banding pattern. In PCR based work we deal with DNA fragments that are often in .1-1 kb range, vs. genomic DNA fingerprints in which a chromosome of 5,000,000 kb is restricted into thousands of fragments and the fragments are resolved and analyzed. Arakawa's work has no standing here. The intention here is not to patent the use of ethidium bromide and the UV light for detecting DNA fragments, the claim is for their use (and use of similar dyes) in visualizing the MRF-RFLP pattern since previously to our invention it was impossible to generate and visualize chromosomal RFLP patterns in <18kb size range using these stains.

i. Article 16: Claims 13 and 22 are rejected under 35 U.S.C. 103(a) as being unpatentable over Preston et al. (1994) as applied to claims 1-4, 6, 11, 12, 16, 19, 21, and 26 above, and further in view of Clayton et. Al., (19930 and Stratagene Catalogue (1994, page 211).

Dr. Lu, I respectfully request that the claims be reinstated in light of the fact that the Preston's standing was discussed in detail in previous sections. I will now discuss the applicability of the Claytons work to the MRF invention. Once again a distinction should be made between the methods used for separating PCR amplicons and RFLP analysis of genomic DNA. The Clayton's paper discusses the of single or double digestion for cutting small PCR amplicons into a few fragments to allow for identifying any differences in these short pieces of DNA. In MRF we use perform the RFLP analysis on total genomic DNA. We have invented a method that allows for visualizing complex RFLP patterns by restriction enzyme digestion of the entire chromosome which is at least a 1000 times larger than the PCR amplicons that were analyzed by Clayton et al. My claim is not to have invented the double enzyme digestion, I have simply found a new use for double enzyme digestion that produces an **unexpected** result when applied to chromosomal DNA, by

producing an RFLP pattern that divides the isolates into groups of clonal origin.

Conclusion

For all the reasons given above, this application is now submitted to contain claims which define a novel, patentable, and truly valuable invention. The use of the methodology has resulted in early detection of a multistate outbreaks of infectious diseases. Presently the MRF is the most senstive subtyping method that can provide results for strains that are untypable by PFGE.

Hence allowance of this application, as amended is respectfully submitted to be proper and is respectfully solicited. In view of the above remarks, reconsideration and allowance of the claims are respectfully requested.

Should there be any remaining issues or topics which should be addressed, the applicant respectfully requests that the examiner contact the undersigned, by telephone: (206) 660-5090.

December 20, 2001

Samadpour

MANSOUR SAMADPOUR